Programmed Cell Death in the Apical Ganglion During Larval Metamorphosis of the Marine Mollusc *Ilyanassa obsoleta*

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Abstract:

The apical ganglion (AG) of larval caenogastropods, such as *Ilyanassa obsoleta*, houses a sensory organ, contains five serotonergic neurons, innervates the muscular and ciliary components of the velum, and sends neurites into a neuropil that lies atop the cerebral commissure. During metamorphosis, the AG is lost. This loss had been postulated to occur through some form of programmed cell death (PCD), but it is possible for cells within the AG to be respecified or to migrate into adjacent ganglia. Evidence from histological sections is supported by results from a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which indicate that cells of the AG degenerate by PCD. PCD occurs after metamorphic induction by serotonin or by inhibition of nitric oxide synthase (NOS) activity. Cellular degeneration and nuclear condensation and loss were observed within 12 h of metamorphic induction by NOS inhibition and occur before loss of the velar lobes, the ciliated tissue used for larval swimming and feeding. Velar disintegration happens more rapidly after metamorphic induction by serotonin than by 7-nitroinda-zole, a NOS inhibitor. Loss of the AG was complete by 72 h after induction. Spontaneous loss of the AG in older competent larvae may arise from a natural decrease in endogenous NOS activity, giving rise to the tendency of aging larvae to display spontaneous metamorphosis in culture.

Article:

Introduction

The apical ganglion (AG), which contains the cephalic or apical sensory organ (Bonar, 1978b; Page and Parries, 2000; Page, 2002b; Nielsen, 2004) is derived from the trochophore apical tuft, a larval structure that has historically been described as a sensory organ (Raven, 1966; Page and Parries, 2000; Ruthensteiner and Schaefer, 2002; Dickinson and Croll, 2003). Results of experiments on competent larvae of the nudibranch *Phestilla sibogae* indicate that the AG can detect dissolved metamorphic cues (Hadfield *et al.*, 2000). Because efferent fibers from the AG innervate velar musculature and ciliation, this ganglion is probably best considered to be a sensorimotor one, as it most likely drives various larval behaviors (Marois and Carew, 1997a, 1997c; Page, 2002b). In gastropods, where several species have been examined, the unpaired AG disappears during larval metamorphosis (Barlow and Truman, 1992; Lin and Leise, 1996a; Marois and Carew, 1997b). In the abalone *Haliotis rufescens* and the sea hare *Aplysia califomica*, its neurons are presumed to disappear through a form of programmed cell death, rather than by incorporation into the subjacent cerebral ganglia (Barlow and Truman, 1992; Marois and Carew, 1997b). In *Ilyanassa*, at the onset of metamorphosis, the neuropil of the AG begins to shrink and the ganglion is no longer visible by 4 days after induction (Lin and Leise, 1996a), but we are not aware of any investigation into the mechanism or timing of AG loss in this molluscan class.

Typically, cells that are destined to die during organismal development do so by a gene-activated process known as programmed cell death (PCD) (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). As an organism matures and body parts change function and form, cells are added or removed. Such changes are prominent in vertebrate nervous systems, which have evolved with an initial overproduction of cells. Neurons that fail to establish functional synaptic connections are removed by apoptosis (Heemels, 2000), the most common form of PCD.

Apoptotic cells are characterized by chromatin condensation, fragmentation of DNA and proteins, cell shrinkage, and membrane blebbing (Kerr *et al.*, 1972; Sastry and Rao, 2000). However, studies on some model systems have revealed cells undergoing PCD that do not conform to the original descriptions of apoptosis (Sperandio *et al.*, 2000). In the invertebrates, one well-known example of non-apoptotic cell death occurs in the intersegmental muscle cells of the hawkmoth *Manduca sexta.* As they degenerate, these muscles display membrane wrinkling and intact genomic DNA (Schwartz *et al.*, 1993), features that differ from those included in classical descriptions of apoptotic cells (Kerr *et al.*, 1972; Wyllie *et al.*, 1980).

Pathways for alternate forms of PCD have yet to be elucidated in as much detail as apoptotic ones. The different forms of PCD are all gene-activated but are distinguished by different morphologies, biochemical pathways, and cellular responses to apoptotic inhibitors (Schwartz *et al.*, 1993; Sperandio *et al.*, 2000; Leist and Jaattela, 2001). After activation, apoptotic pathways usually induce release of death-regulatory molecules from cytoplasmic mitochondria (Boyd and Cadenas, 2002; Claveria and Torres, 2003; Kroe-mer, 2003). However, cell death can ultimately occur through different mechanisms, including both caspase-dependent and caspase-independent processes (Green, 1998; Lockshin and Zakeri, 2002; Abraham and Shaham, 2004). Cell death that arises through a caspase-independent path-way can ensue after a sequence of cellular changes that includes a reduction or delay in chromatin condensation and increased cytoplasmic vacuolization (Oppenheim *et al.*, 2001; Cregan *et al.*, 2002; Abraham and Shaham, 2004). But for both caspase-dependent and caspase-independent apoptosis, nitric oxide (NO) has been implicated as a potential regulatory substance (Dennis and Bennett, 2003).

Endogenous NO can inhibit PCD by direct S-nitrosation of either active caspases or their proenzymes (Kim *et al.*, 1997; Liu and Stamler, 1999; Kolb, 2000; Boyd and Cadenas, 2002; Wang *et al.*, 2002; Brune, 2003). Conversely, under appropriate cellular conditions, NO and its derivatives can interact with mitochondrial proteins, leading to damage in the ATP-generating system and increased permeability of mitochondrial membranes. Ultimately, this in-creased membrane permeability can result in the release of cytochrome *c* and apoptogenic proteins that will lead to cell death (Uehara *et al.*, 1999; Joza *et al.*, 2001; Boyd and Cadenas, 2002; Brown and I3orutaite, 2002; Dennis and Bennett, 2003; Kroemer, 2003). Such opposing nitrergic mechanisms depend heavily on the site, duration, and amount of NO produced (Nicotera *et al.*, 1997; Li and Billiar, 2000), with low levels of endogenous NO generally being responsible for anti-apoptotic effects and excess production during pathological conditions leading to cell death (Fiscus, 2002).

Since its initial identification as endothelium-derived relaxing factor (Furchgott and Vanhoutte, 1989; Moncada *et al.;* 1989, Ignarro, 1990), the gaseous neurotransmitter NO has been shown to have a broad phylogenetic distribution (Jacklet, 1997) and to be active in processes beyond PCD, including the regulation of neurite outgrowth (Wu *et al.*, 1994; Cogen and Cohen-Cory, 2000; Seidel and Bicker, 2000) and growth cone activity (Hess *et al.*, 1993; Renteria and Constantine-Paton, 1995; Van Wagenen and Rehder, 2001), synaptogenesis (Gibbs and Truman, 1998; Gibbs, 2001; Gibbs *et al.*, 2001), and modulation of neurogenesis and neuronal differentiation (Peunova and Enikolopov, 1995; Kuzin *et al.*, 1996; Enikolopov *et al.*, 1999; Kuzin *et al.*, 2000; Murillo-Carretero *et al.*, 2002; Moreno-Lopez *et al.*, 2004). NO has also been identified in larvae of several invertebrates through the use of NADPH diaphorase (NADPHd) histochemistry (Lin and Leise, 1996b; Meleshkevitch *et al.*, 1997; Serfözö *et al.*, 1998). In *Ilyanassa obsoleta*, larval metamorphosis can be induced by serotonin (Levantine and Bonar, 1986; Couper and Leise, 1996) or the injection of nitric oxide synthase (NOS) inhibitors (Froggett and Leise, 1999), but not by application of NO-donors. These results indicate that in this animal, NO production is required for the maintenance of the larval state. Because NO is expressed in almost all of the neurons of the AG in larval *Ilyanassa* (Thavaradhara and Leise, 2001), because levels of NADPHd activity appear to be maximal at metamorphic competence (Lin and Leise, 1996b), and because NO can inhibit forms of PCD (Peunova *et al.*, 1996; Kolb, 2000; Thippeswamy *et al.*, 2001; Fiscus, 2002; Brune, 2003), we hypothesized that NO might be inhibiting metamorphosis in competent larvae by preventing cell death in the AG.

During metamorphosis in gastropod larvae, degeneration occurs in a variety of tissues, including the musculature, nerves, and epidermis of the velum (Bonar and Hadfield, 1974; Bonar, 1978a); gland cells of the

foot (D'Asaro, 1965); the larval kidneys; and regions of the digestive tract (Bonar, 1978a; Bickell *et al.,* 1981). Until recently, most authors working on molluscan metamorphosis did not specifically address the issue of PCD, which is a major hallmark of developing nervous systems. As such, it is important to determine the extent to which this process occurs in the Mollusca (Marois and Carew, 1990). Because of the complexity of the PCD pathway and potentially valid alternate hypotheses, it was unclear to us which form of PCD might occur during the metamorphic loss of the AG. Whether the AG was lost early or late in the metamorphic process was also unknown. Thus, we examined larvae histologically for the presence of the AG and used a TUNEL assay and Hoechst 33342 staining in our attempts to identify dying cells. We examined competent and metamorphosing larvae at 12-h intervals for 4 days after metamorphic induction. Our results demonstrate that loss of the AG can begin within 12 h of metamorphic induction and before the velar lobes degenerate; that loss is dependent upon NOS inhibition; and that the AG completely disappears by 72 h after induction, not 96 h as previously reported by Lin and Leise (1996a).

Materials and Methods

Adult specimens of *Ilyanassa obsoleta* (Say, 1922; synonymous with *Nassarius obsoletus)* were collected from intertidal mud flats along the North Carolina coast, kept in three seawater aquaria, and fed frozen fish daily. The snails laid egg capsules for at least 9 months of the year, typically December through August. Detailed descriptions of larval culture care have been published previously (Couper and Leise, 1996; Leise, 1996; Froggett and Leise, 1999; Leise *et al.,* 2001; Thavaradhara and Leise, 2001). Briefly, egg cap-sules were collected several times a week from laboratory populations of breeding adults. Newly hatched larvae were placed in an airlift system in a 1:1 mixture of 0.2-μm-filtered natural seawater and Instant Ocean with penicillin and streptomycin antibiotics (Couper and Leise, 1996). Larvae were fed daily with a 1:1 mixture of the algae *Isochrysis galbana* and *Dunaliella tertiolecta.* Larvae were reared at a density of about 1 larva per 1.5 ml of culture fluid at ambient temperatures, between 24 °C and 26 °C, until metamorphically competent. In larval *L obsoleta,* the competent period can begin as early as 16 days after hatching and can continue for over 2 months in culture (unpubl. obs.). Larvae will continue to grow in culture for several months, as long as they are fed, to a maximum of about 950 gm (Scheltema, 1967). In lieu of inspecting each animal for evidence of a propodium (Dickinson and Croll, 2003), we estimated metamorphic competence by measuring maximum shell length, defining 100% of larval development (and capability to metamorphose) as a shell length of 600 μm, which occurs around 20 days after hatching (Scheltema, 1961, 1962; Thavaradhara and Leise, 2001). It is thus possible to experiment upon animals with shell lengths smaller or larger than 600 gm and beyond 100% of larval development. At 100% of development, over 85% of larvae are typically competent and will metamorphose in response to 0.1 m*M* serotonin (5-HT) within 48 h, but will not metamorphose spontaneously. We used animals from cultures with average shell lengths of 590-610 μm. To find this average, 10-15 animals were randomly selected from each culture and measured under a dissecting microscope at 40 ×.

Induction of metamorphosis

Each culture of about 550 competent larvae was split into two groups and induced to metamorphose 12 h apart by bath application of either 0.1 m*M* 5-HT or 0.2 m*M* 7-nitroinda-zole (7-NI) (Cayman Chemical Co.). 7-NI, a selective NOS inhibitor (Bush and Pollack, 2000), was chosen because it triggers metamorphosis in bath application at rates similar to those induced by 5-HT after 48 h, as long as it is stored frozen at —20 °C (Durham, 2002; Leise *et al.,* 2004). Animals induced with 5-HT remained in this solution for 12 h and were then placed in 0.2-μm-filtered Instant Ocean (FI0). Animals induced with 7-NI remained in it for 48 h and were then placed in FIO. After removal of either inductive solution, FIO solutions were changed daily. Metamorphosing larvae and juveniles were removed at 12-h intervals up to 96 h and prepared for subsequent embedding in soft Spurr's resin (Spurr, 1969) or JB-4 glycol methacrylate (GMA; Electron Microscopy Sciences). Animals re-moved at 12 or 24 h after induction were initially separated into six groups according to their morphological progress through metamorphosis. The six groups were (1) larvae, (2) larvae with velar lobes losing cilia, (3) larvae with velar lobes without cilia, (4) larvae with partial velar lobes, (5) larvae with cephalic mounds of tissue, and (6) juveniles. Initially, we did not know when PCD might occur during the metamorphic process, so specimens examined at 24-96 h were juveniles unless otherwise indicated in the figure

legends. Once we realized that PCD began early in the metamorphic process, we examined intact larvae at 12 and 24 h past induction.

Light microscopy

Larvae to be examined with routine histological methods were anesthetized for 7 min in a high Mg^{2+} (110 mM)/high Ca^{2+} (25 mA/1) solution to relax and immobilize them (Norekian and Satterlie, 1993), then fixed at 4 °C for 3 h in a 2.5% solution of Millonig's phosphate-buffered gluteraldehyde, with 0.14 *M* NaC1 (Cloney and Florey, 1968). Specimens were decalcified overnight in a solution of 10% EDTA mixed 1:1 with the above fixative solution and post-fixed at 4 °C for 30 min in a 2% solution of osmium tetroxide in 1.25% NaHCO3 (Leise and Cloney, 1982). They were then dehydrated at 0 °C in an ethanol series and rinsed at 0 °C in pure acetone, three times, for 10 min each. Larvae were infiltrated at 4 °C with multiple solutions of acetone/pure soft Spurr's (Spurr, 1969) resin (2:1, 1:1, and 1:2) for 30 min each, and lastly infiltrated at 4 °C overnight in pure Spurr's resin (Polysciences, Inc.). Specimens were embedded in flat embedding molds for 16 h at 60 °C. Embedded specimens were sectioned at 5 μm, placed on slides coated with 3'aminopropyl-triethoxy silane (TESPA) (Ben-Sasson *et al.*, 1995), stained with Richardson's stain (Richardson *et al.*, 1960) diluted 1:9 with distilled water, mounted in high-viscosity immersion oil (Cargille Laboratory, Type NVH) or Permount (Electron Microscopy Sciences), and examined with an Olympus BH-2 compound microscope. Some larvae that were embedded in GMA as described below for detection of PCD were also sectioned at 3 pm, and alternate sections were stained with Richardson's stain, diluted 1:24 with distilled dH_20.

Sections were photographed with either a Kodak DCS 420c or a Canon EOS 10D digital camera mounted on the above compound microscope connected to either a Power Macintosh 7300/180 or a Dell Dimension 8300 running Windows XP (Microsoft Corp.), respectively. Images were captured on appropriate Canon or Kodak utility software packages, contrast and brightness were optimized in Adobe Photoshop 8.0 (Adobe Systems, Inc.), and image files were then stored in digital format on compact disks and printed on a Kodak 8500 digital photo printer.

Degenerating cells in the AG of metamorphosing larval were counted on consecutive sections. Sections were traced from video images captured with a Javelin MOS solid state video camera linked to an Ikegami color monitor. Because cells could appear on three or four adjacent sections, drawings were examined to eliminate counting individual cells more than once. When in doubt, we recorded the minimal number of degenerating neurons.

Detection of programmed cell death

As previously mentioned, larvae were induced to metamorphose and grouped at 12 and 24 h after metamorphic induction. These animals had been decalcified overnight in low-pH seawater (Cavanaugh, 1956) at ambient temperature before metamorphic induction. At 12 and 24 h, each group of larvae was anesthetized as previously described and fixed at 4 °C, overnight, in 4% paraformaldehyde in 0.2 *M* Millonig's phosphate buffer. Next, specimens were de-hydrated in an ethanol series and infiltrated at 4 °C in two mixtures of absolute ethanol/GMA catalyzed infiltration solution A (1:1 and 1:2) for 3.0 min each, and then infiltrated at 4 °C overnight in JB-4 GMA catalyzed infiltration solution A. Individual specimens were placed into the tips of 00 Beem capsules, and the capsules were filled with the complete GMA solution. These were polymerized at ambient temperature overnight. Specimens were sectioned at 3 pm; alternate sections were placed on slides coated with TESPA and then analyzed using the TUNEL assay (Dead End Colorimetric Apoptosis Detection System Kit, Pro-mega Corp. #G7130) or stained with Richardson's stain, diluted 1:24 in distilled dH_20. The assay was conducted according to the directions provided in the kit. Permeabilization with 20 μg/m1 proteinase K was optimized at 18 min. Equilibration and blocking were done at the maximum recommended times. Sections were mounted in Permount and observed with standard light microscopy. Positive and negative controls were also conducted as directed by the kit's protocol. Sections were photographed and images treated and stored as described above.

Hoechst 33342 staining

Sections were also treated with Hoechst 33342 stain (Molecular Probes Inc., catalog #H-1399) in an additional attempt to detect DNA fragmentation during early stages of PCD. Specimens were embedded in GMA and mounted on slides for this procedure. Sections were rinsed at ambient temperature in 0.1 *M* phosphate buffer solution (PBS) for 15 mins. Slides with 3-µm sections were incubated in a coplin jar for 1 h in a 1-µg/m1 solution of Hoechst 33342 stain, in 0.1 *M* PBS, at ambient temperature. Slides with 5-µm sections were incubated in the same way for 1.5 h. Sections were rinsed three times for 10 min with 0.1 *M* PBS and air-dried (Masseau *et al.,* 2002). Once dry, the sections were mounted with ProLong Antifade (Molecular Probes, catalog # P-7481) and viewed under an Olympus Fluoview 500 laser scanning confocal unit mounted on an inverted Olympus IX81 motorized microscope at excitation and emission peaks of 352 nm and 461 nm. Alternatively, sections cut earlier in the project period were viewed by epifluorescence under a BH-60 Olympus compound microscope with a DAPI filter cube with excitation and emission peaks at 360 nm and 460 nm, respectively. AG were examined for the presence of nuclei that showed a color shift towards a more blue fluorescence, indicative of fragmented DNA, in comparison to nuclei with intact DNA (Sun *et al.,* 1992). Images were captured with the Olympus Fluoview FV500 software, ver. 4.3a, stored on CDs, and again manipulated in Adobe Photoshop 8.0 to maximize contrast.

Comparison of metamorphic induction with serotonin and 7-nitroindazole

Rates of metamorphosis in 5-HT and 7-NI were com-pared in standard bath application experiments (Couper and Leise, 1996; Froggett and Leise, 1999). Briefly, experiments were conducted in 24-well, plastic, untreated, Falcon tissue culture plates with 2 ml of experimental solution and five larvae per well. The typical negative control was FIO. Each treatment was replicated 18 times for a total of 90 larvae per treatment. Larvae remained in the experimental solutions, 0.1 m*M* 5-HT and 0.2 m*M* 7-NI, for 48 h. Numbers of larvae and "juveniles" (individuals without velar lobes or that had lost more than half of their velar tissue) were counted at 24 and 48 h. Results were tested for statistical significance *(P* = 0.05) in a two-way chi-square contingency table (Zar, 1974; Sokal and Rohlf, 1981). Raw percentage data were normalized by an arcsine transformation, and standard deviations were calculated from the transformed data. Data were transformed back to percentages for graphing. Graphs were produced in Graphpad Prism 4.02 (Graphpad Soft-ware, Inc.).

Results

A major anatomical difference between a competent larva and a juvenile of *Ilyanassa obsoleta* is the presence of the apical ganglion (AG) in the larval nervous system (Lin and Leise, 1996a). In competent larvae, the AG is a cluster of relatively large cells that lie above a subjacent neuropil (Fig. 1; Lin and Leise, 1996a). Induction of larval metamorphosis by serotonin (5-HT) or 7-nitroindazole (7-NI) induced distinct changes in cellular morphology within this ganglion.

Within 24 h of metamorphic induction by 5-HT or 7-NI, most cells in the AG *(e.g.,* 73% after 7-NI induction, *n* = 4) showed signs of programmed cell death (PCD) (Fig. 2A, B). Higher magnification images at this time point of juvenile specimens resulting from serotonergic induction displayed cytoplasmic vesicles and cells resembling macrophages, along with cellular debris associated with phagocytic activity (Fig. 2C). Similar individuals induced with 7-NI showed signs of cellular degeneration in the AG (Fig. 2D). Because both such juvenile specimens retained some nucleated cells in the AG, we performed a TUNEL assay and stained sections of the AG with Hoechst 33342 in metamorphosing larvae. DNA fragmentation or loss of chromatin in the AG of these specimens would indicate PCD. The presence of two TUNEL-positive cells with pyknotic nuclei in the AG (Fig. 2E) supported our hypothesis that cells in the AG are undergoing PCD. The corresponding alternate section (Fig. 2F) verified that the dying cells are within the AG. Results of the positive control for the TUNEL assay (procedure conducted with DNase) and a negative control (omission of the terminal deoxynucleotidyl transferase enzyme) validated our findings (data not shown). An examination of the AG in intact larvae 12 h after metamorphic induction by 7-NI revealed that 57% of its cells (*n* = 6) were undergoing PCD (Fig. 2J). A cursory examination of two 7-NI-induced larvae at the same 12-h time period, but at a later stage of metamorphosis, with velar lobes losing cilia, yielded a higher percentage (78%) of degenerating cells, as might be expected. Although the onset of PCD appeared to be more rapid with 7-NI than with 5-HT *(c.f.,* Fig. 2A—

D), similar numbers of cells in the AG were apoptotic. Surprisingly, we detected no color-shifted nuclei in cells of the AG that were stained with Hoechst 33342 at 24 h (Fig. 2G) nor at 12 hours past induction with 7-NI (Fig. 21).

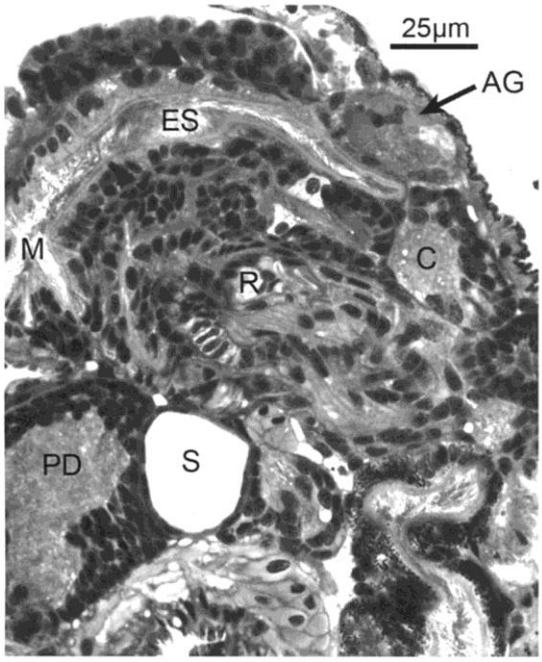

Figure 1. Sagittal 5-μm section through a competent larva displays an intact apical ganglion (AG). The cerebral commissure is not visible as this is not a midline section. ×475. C, cerebral ganglion; ES, esophagus; M, mouth; PD, pedal ganglion; R, radula; S, statocyst.

To determine whether there was a difference in metamorphic induction by 5-HT or 7-NI, we compared the numbers of larvae in which metamorphosis was triggered by each method at 24 and 48 h. At both times, metamorphic induction by 5-HT typically resulted in significantly more larvae with visible signs of metamorphosis, including loss of velar ciliation or velar lobe tissue, than did induction by 7-NI (Fig. 3).

At 36 h after induction with either 7-NI or 5-HT, pyknotic nuclei and vesiculation within the neuropil of the AG suggested continuing PCD activity (Fig. 4A, B). By 48 h after induction, pyknotic nuclei and a loss of cellular integrity in the AG, as indicated by the presence of large cytoplasmic vesicles (Fig. 4C, D), continued to be visible. At 60 h after induction by either method, cells of the AG no longer contained recognizable nuclear material. The majority of the nuclei of the cells in the AG had disappeared, leaving large cytoplasmic regions (Fig. 4E, F). By 72 h after metamorphic induction, the AG had disappeared in animals induced with 7-NI or 5-HT (Fig. 4G—J). There was nothing left but cellular debris scattered above the cerebral commissure (CC).

After about 3 weeks in culture, when the average lengths of animals are above 600 μm (100% of larval development), larvae of *L obsoleta* lose specificity and begin to metamorphose spontaneously on the walls of the culture beakers. We examined such aged competent larvae, with intact and functional velar lobes, from cultures at 105%-110% of larval development. These animals displayed the beginnings of cellular breakdown in the AG without exposure to a metamorphic inducer (Fig. 5A). When exposed to Hoechst 33342 dye, intact nuclei in the AG were stained, but no color-shifted nuclei were evident. Many cells in AG with degenerating nuclei did not absorb the Hoechst stain (Fig. 5B,C).

Discussion

In *Ilyanassa* and other gastropod larvae, neuronal cell death has been implicated in the loss of the larval apical ganglion (AG), but much of the evidence for this arises from examinations of the ontogeny of individual immunoreactive neurons (Barlow and Truman, 1992; Marois and Carew, 1997b; Page, 2002b; Dickinson and Croll, 2003). During development, neurons can change neurotransmitter expression (Kandel *et al.*, 2000): for the limpet *Tectura scutum*, Page (2002a) reported the retention of an AG sensory neuron that loses its

serotonergic immunoreactivity; in the opisthobranch *Aplysia californica*, Marois and Carew (1997b) documented degeneration of the serotonergic neurons in the AG. Our results indicate that in *Ilyanassa*, loss of the AG is complete by 72 h after metamorphic induction, although this is not the end of this developmental process, as young juveniles are apparently incapable of feeding until their 4th day past induction (pers. obs.). Thus, the metamorphic process, while unidirectional, is a rather lengthy one in this species, taking several days before the young juvenile is fully functional. Reproductive activity requires further maturation, about 3-4 years (Curtis and Hurd, 1983; Curtis, 1995).

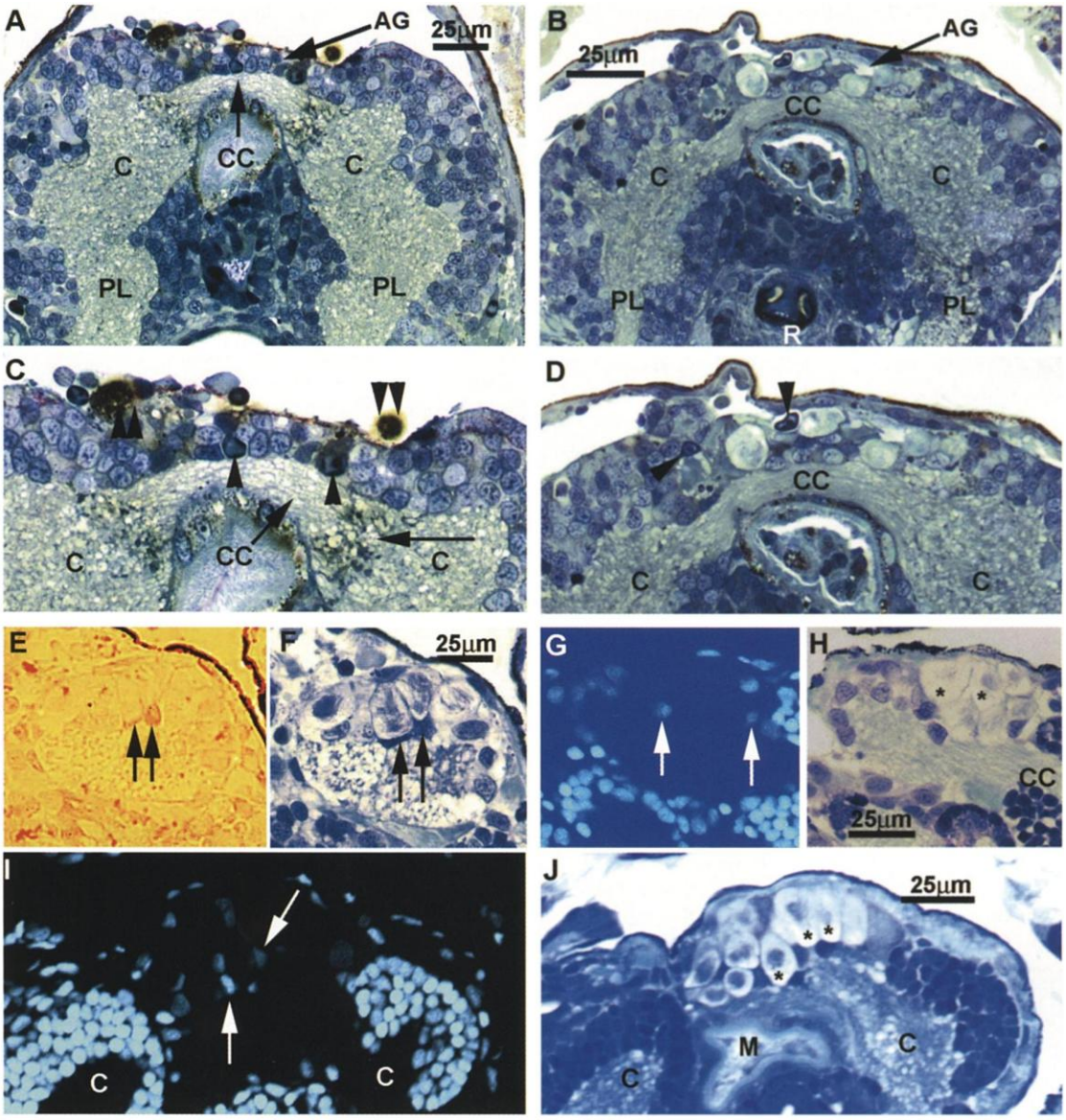

Figure 2. Metamorphosed juveniles (A–D) and larvae (E–J) display degeneration of the apical ganglion (AG) at 24 or 12 h after metamorphic induction. (A, B) Young juveniles 24 h past induction by 5-HT (A) or 7-NI (B). ×370, ×500. (C, D) Higher magnification of the AG in A and B, respectively, displaying pyknotic nuclei (single arrowheads in C and D). Cells resembling macrophages (double arrowheads in C) occur in the remnants of the AG lying above the cerebral commissure (CC). Right arrow (in C) indicates cellular debris associated with phagocytic activity. ×975, ×650. (E) TUNEL assay and (F) adjacent section of an AG at high magnification from a metamorphosing larva with partial velar lobes, 24 h after induction with 5-HT. Arrows indicate TUNEL-positive nuclei (E) that stain intensely with Richardson's method (Richardson *et al.,* 1960) in the adjacent section (F). These nuclei most likely contain fragmented DNA. ×320, ×330. (G) Epifluorescent image of an AG at high magnification stained with Hoechst 33342, and an adjacent section (H) stained by Richardson's method (1960) from a larva with intact velar lobes at 24 h after induction by 7-NI. We observed no color-shifted nuclei with the Hoechst stain, relatively few stained nuclei (arrows in G), and large cellular remnants (asterisks in H) in the AG. ×400. (I) Confocal image of Hoechst staining and an adjacent section (J) from a larva with intact velar lobes 12 h past induction with 7-NI. Again, we saw no color shift with the Hoechst's stain and a relative paucity of dyed nuclei in the AG (arrows in I). (J) Many cells in the AG have condensed chromatin and little nuclear or cytoplasmic structure (asterisks) when stained by Richardson's method. ×440. C, cerebral ganglion; M, mouth; PL, pleural ganglion; R, radula.

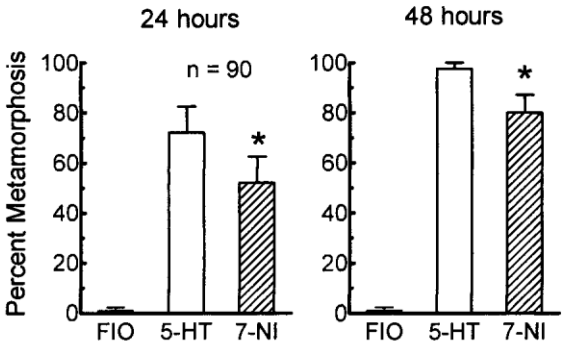

Figure 3. Rates of metamorphosis after 5-HT or 7-NI induction. 5-HT induces more larvae, as indicated by gross morphology. Animals with characteristics of juveniles or that had lost more than half of their velar tissue were counted as metamorphosed individuals. Rates of metamorphosis after 5-HT and 7-NI induction were significantly different (asterisks) at both 24 and 48 h ($\chi^2_{0.005(1)} = 7.58$ and 14.2, respectively). FIO, control in filtered Instant Ocean.

Support for our hypothesis, that programmed cell death (PCD) in the AG is regulated by nitric oxide, is based on several series of experiments. NO-donors can reduce rates of serotonergically induced metamorphosis, while injection of NOS inhibitors, such as N-nitro-L-arginine methyl ester (L-NAME) or N-methyl-L-arginine acetate (L-NMMA), initiates metamorphosis (Froggett and Leise, 1999). Bath application of another NOS inhibitor, 7-nitroindazole (7-NI), also triggers this process (Leise *et al.,* 2004). As reported here, NOS inhibition initiates degeneration in a majority of the cells of the AG. Results of our light microscopic investigations and TUNEL assays of metamorphosing larvae indicate that PCD in the AG begins within 12 h of exposure to a metamorphic inducer, before velar loss, and that the AG is completely absent by 72 h after induction, not 96 h as previously reported (Lin and Leise, 1996a).

In our attempts to support the idea that the cells of the AG in *L obsoleta* die through a form of PCD, we used a TUNEL assay and Hoechst 33342 staining, in which positive results indicate fragmentation of nuclear DNA. We chose DNA destruction as our hallmark of PCD because it has been detected in many forms of PCD (Hara *et al.,* 2004; Rodriguez and Schaper, 2005). At 24 h past metamorphic induction, we detected some DNA breakup with a TUNEL assay in larvae with partial velar lobes. However, at no time point, from 12 h past induction or later, did the Hoechst 33342 staining show any significant color shift (Fig. 2). We interpret the lack of Hoechst color-shifted nuclei—indeed, the lack of any Hoechst 33342 staining in many degenerating nuclei, as well as the relative paucity of TUNEL-positive nuclei—as indications that DNA fragmentation has probably occurred before our 12-h time point. However, there are alternate possibilities. There is still disagreement in the literature over the duration of the cell death event and the timing of DNA fragmentation during this phenomenon; the discrepancies may reflect different experimental paradigms or cell populations. Cell death is usually completed in 1 to 24 h (Wyllie, 1994; Studzinski, 1999; Suzuki *et al.,* 2001; Rodriguez and Schaper, 2005). Some authors have linked DNA fragmentation to pyknotic nuclear morphology (Suzuki *et al.,* 2001), but most apical ganglionic nuclei were not pyknotic, so these cells were either well past that stage at our examination or were degenerating through a pathway that does not include nuclear condensation (Oppenheim *et al.,* 2001; Abraham and Shaham, 2004). Several authors have also remarked upon the difficulty of detecting results with specific tests when the dying cell population is small, as it is in the AG, and when the time course of events is unknown (Wyllie, 1994; Studzinski, 1999; Hacker, 2000). As they suggest, the various forms of PCD can result in distinctive cellular morphologies and in different delay pe-riods between initiation and the onset of cellular destruction (Green, 1998; Cregan *et al.,* 2002; Lockshin and Zakeri, 2002). Studies of caspase-independent apoptosis have demonstrated a lower number of TUNEL-positive cells and less visible DNA fragmentation in mammalian neurons (Oppenheim *et al.,* 2001; Abraham and Shaham, 2004). This could also explain the results of our studies on molluscan larvae. The presence of large anuclear cytoplasmic remnants in our sections for up to 60 h also suggests that either the final engulfment and digestion phase of the cell death process in *Ilyanassa* is relatively long or cells in the AG die in waves. Both the time course of events and the

particular PCD pathway involved require further exploration, but our results indicate that in the AG, cell death begins early in the metamorphic process.

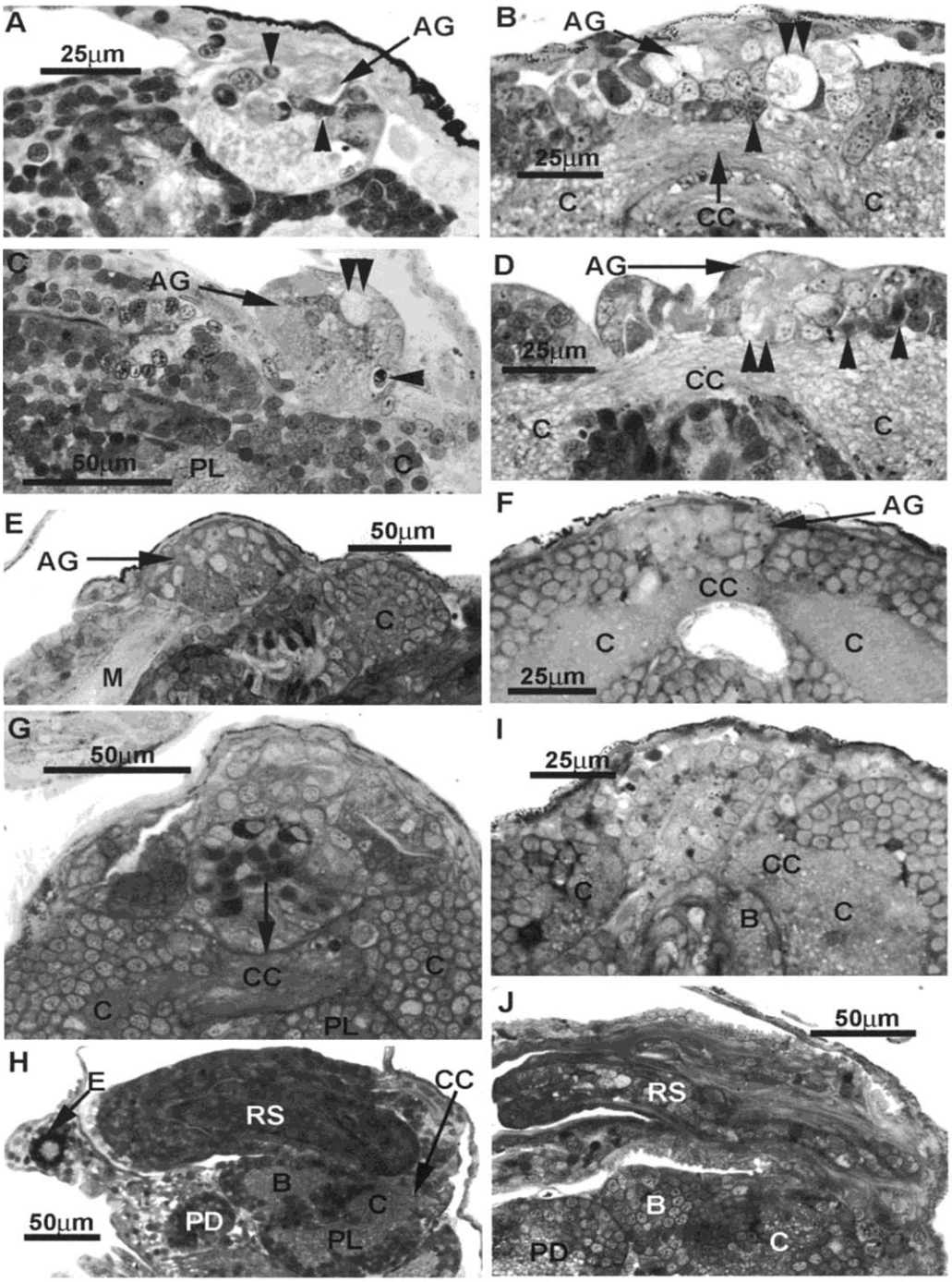

Figure 4. Sagittal (A, C, E, H, J) and transverse (B, D, F, G, I) 5-μm sections of apical ganglia (AG) in specimens embedded in soft Spurr's (1969) resin and stained with Richardson's method (Richardson *et al.,* 1960). Sagittal (A) and transverse (B) sections of larvae 36 h after induction with 5-HT (A) or 7-NI (B). Some nuclei in the apical ganglia are pyknotic (single arrowheads), and larvae induced with 7-NI display evidence of loss of cellular integrity, as indicated by the appearance of large vesicles (double arrowheads in B). Sagittal (C) and transverse (D) sections of larvae 48 h after induction with 5-HT (C) or 7-NI (D). AG in C has pyknotic nuclei (single arrowhead), while dark (brown in original stained sections) cellular regions in larvae induced with 7-NI suggest local phagocytic activity (single arrowheads in D). AG in both sections display more vesiculated cellular material among the somata (double arrowheads). By 60 h after metamorphic induction, whether by 5-HT (E) or 7-NI (F), most apical ganglionic nuclei have disappeared, leaving large cytoplasmic remnants. At 72 h after metamorphic induction, again by either 5-HT (G, H) or 7-NI (I, J) the AG is no longer recognizable. Furthermore, the cerebral commissure (CC) is now separated from overlying tissue, that is not the AG, by a distinct glial layer (arrow in G). Transverse (G, I) and near midline (H, J) sections are shown for comparison. Anterior is to the left in all sagittal sections, and dorsal is up in all sections. A, ×590; B, ×500; C, ×410; D, ×480; E, ×310; F, ×510; G, ×470; H, ×240; I, ×475; J, ×320. B, buccal ganglion; C, cerebral ganglion; E, eyespot; M, mouth; PD, pedal ganglion; PL, pleural ganglion; RS, radular sao.

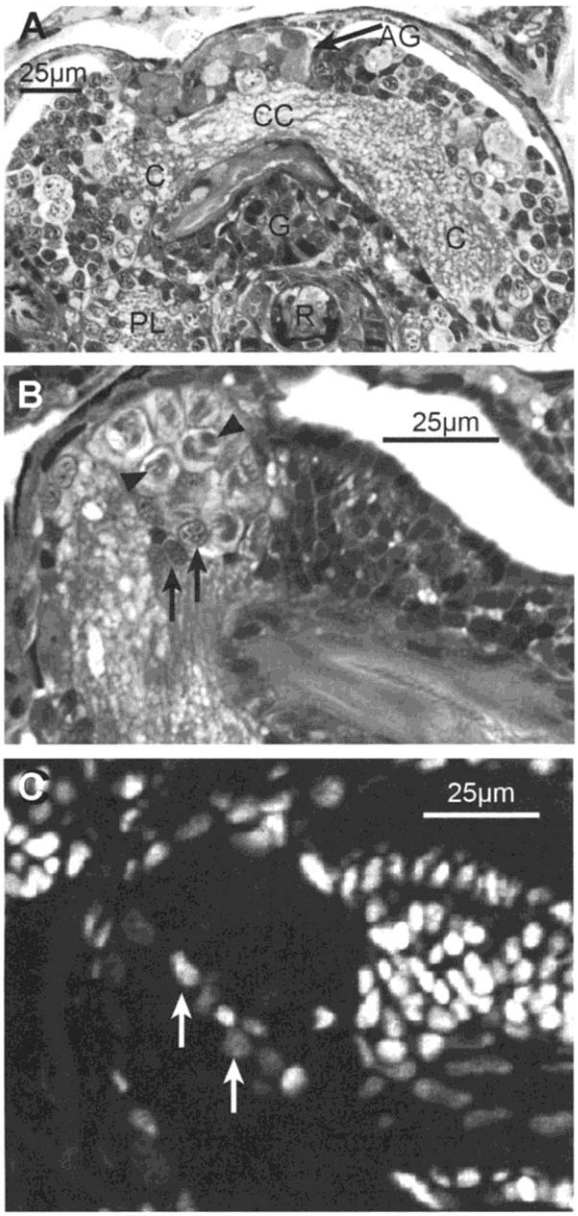

Figure 5. Larvae at 105% (A) and 110% (B, C) of development. (A) Transverse section of an aged competent larva embedded in soft Spurr's resin and stained with Richardson's method (Richardson *et al.*, 1960). The apical ganglion (AG) shows signs of cellular degeneration without prior exposure to a metamorphic inducer. ×320. (B, C) Adjacent obliquely transverse sections of an aged competent larva embedded in JB-4 glycol methacrylate and stained with Richardson's (B) or Hoechst 33342 dye (C). Intact nuclei in the AG (arrows in B, C) stain with the Hoechst dye, whereas degenerating nuclei (arrowheads in B) do not. ×630. C, cerebral ganglion; CC, cerebral commissure; PL, pleural ganglion; R, radula.

Initial examinations of our sectioned material suggested that internal changes were occurring more rapidly after metamorphic induction with 7-NI than with 5-HT, because we saw more cytoplasmic degeneration in the AG at earlier times with 7-NI (Fig. 2B). Our admittedly limited counts of degenerating neurons in sectioned larvae did not support this idea, but more animals need to be examined to deter-mine the time course of events after application of various metamorphic inducers. We did see more velar loss, at statistically significant rates, after 5-HT induction, which suggests that the linkage between velar loss and internal morphology may depend upon the inductive cue. That velar loss occurs more slowly after direct NOS inhibition suggests that NO actions are downstream of serotonergic events, but more experimentation is needed to understand the molecular pathways that trigger velar loss.

Our results also shed light on the significance of the delay that, in gastropod metamorphosis, can occur between larval exposure to an inductive cue and the loss of the velar lobes. Velar loss has routinely been used as a key indicator of successful metamorphic initiation, yet in *Phestilla sibogae,* for example, at least 10 h elapses between exposure to an appropriate metamorphic cue and the beginnings of velar loss (Koehl and Hadfield, 2004). This lag has not been well understood in mechanistic terms, but our results indicate that cell death pathways in the central nervous system are being activated during this time, well before any outward signs of metamorphosis become visible. How rapidly neural PCD is triggered by metamorphic inducers remains to be determined.

As larval *Ilyanassa* age, they begin to metamorphose spontaneously (Pechenik, 1980). A natural decline in NOS gene expression or enzymatic activity as the competent period progresses could be responsible for the spontaneous metamorphosis seen in laboratory cultures (Pechenik *et al.,* 2002). Our examinations of aging larvae support this idea, but a complete understanding of this natural phenomenon requires further analyses of the regulation of NOS activity and mRNA production, or the translational or post-translational modifications that could lead to lowered rates of NO release.

NO has now been implicated as a negative regulator of larval metamorphosis in representative species of three major invertebrate groups: the Mollusca (Froggett and Leise, 1999; Pechenik *et al.,* 2002), the Echinodermata (Bishop and Brandhorst, 2001), and the Urochrodata (Bishop *et al.,* 2001). In all of the species examined, the loss of larval tissues, including all or part of the larval nervous system, is an important component of the metamorphic process. Bishop and Brandhorst (2003) theorized that nitrergic regulation of metamorphosis may be a plesiomorphic larval characteristic, having evolved as an efficient way to regulate developmental processes in specific tissues or cell clusters (Edelman and Gally, 1994). Apical ganglia are conserved throughout invertebrate phyla (Lacalli, 1994), and we suggest that where such larval ganglia are lost, NO may like-wise be retained as an inhibitor of the cell death process.

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